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ScienceDirect
Enzyme and Microbial Technology 43 (2008) 226–232
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Bioconvertion of spent cellulose sausage casings

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Received 15 August 2007; received in revised form 8 September 2007; accepted 10 September 2007

Abstract

Cellulose sausage cellulose casings are used extensively in the manufacture of sausages in meat packaging. After stripping the meat, spent casings mainly contain cellulose and residual meat juice with salt, nitrate and nitrite. Disposal of spent sausage casings has serious economic and environmental concerns for the sausage industry. This work describes bioconversion of spent cellulose casings (SCC) into enzymes, lactic acid and ethanol by using cellulolytic fungi, lactobacillus and yeasts. The solid substrate cultivation (SSC) of *Trichoderma reesei* RUT C-30 on SCC and blends gave a maximum of 152 filter paper cellulase (FPase) activity and about 100 carboxymethylcellulase activity (CMCase)/g dry weight substrate. The SSC produced enzyme-rich casing with 50 FPase when directly mixed as such with 10% fresh SCC produced over 70 g/l lactic acid using *Lactobacillus plantarum* sp. 14431, and also produced 30 g/l ethanol with *Kluveromyces marxianus* IMB-3 under simultaneous saccharification and fermentation (SSF) conditions.

Keywords: Bioconversion; Cellulose; Enzyme-rich casing; Ethanol; Filter paper cellulase (FPase); Lactic acid; Simultaneous saccharification and fermentation (SSF); Solid substrate cultivation (SSC); Spent cellulose casings (SCC)

1. Introduction

Cellulosic casings are widely used for packing meat in sausage manufacture due to their uniform strength and stability. The casing viscose cellulose is made from highly purified cellulose by solubilization in strong alkali, derivitization and reprecipitation [1]. During sausage manufacture, the meat product is packed into a cellulose casing, twisted to seal the ends, and cooked. After cooking, cellulose casings are peeled from the finished product and disposed by landfill. Annual U.S. production of spent cellulose casings (SCC) currently exceeds 14 million kg [2]. The disposed spent casings mainly contain cellulose and residual meat juice with salt, nitrate and nitrite [2]. The rising cost of landfill fees for disposal of spent cellulose casings is a serious economic concern for the sausage industry. Many researchers have examined the enzymatic hydrolysis and fermentation of native cellulose but few have examined the degradation of regenerated celluloses [3–7]. Utilization of SCC as feed ingredients for ruminants and degradation to increase glucose concentration has been reported [2,3,7]. However, disposal through animals would be practical only if a high fraction of the material is digested and the costs involved are less than traditional feed [2,6,7].

The conversion of lignocellulosics to value-added products such as biofuels and chemical feedstocks holds great potential [8–14]. Enzymatic hydrolysis of lignocellulosic biomass is one of the most efficient routes [8,10–12,15,16]. The high cost of commercial cell wall degrading enzymes and their demand in various industrial applications has caused serious research for more economical alternatives. Hence, low cost hydrolytic enzyme production needed in large quantities is a key to economic success of any bioconversion process involving lignocellulosics.

SCC, a source of waste pure cellulose polymer, can be saccharified efficiently by cellulases. The resulting sugars can be simultaneously fermented to high yielding, value-added chemicals. Such processes could be useful to sausage industry as an important means to the waste disposal problem with potential for revenues from value-added products. Information on such

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doi:10.1016/j.enzmictec.2007.09.005

bioconversion steps of SCC for production of value-added products, however, is scanty.

The objective of this study was to utilize pure cellulose from SCC wastes for cellulase production by SSC. Subsequently the SSC produced enzyme-rich casing substrate (without expensive enzyme extraction) was directly mixed as such with fresh SCC for SSF production of high yields of lactic acid or ethanol.

2. Materials and methods

2.1. Raw material

The SCC was obtained from Klements Sausage Co. (Milwaukee, WI) and stored at −4 °C. Prior to use, they were shredded into approximately 4 cm × 0.4 cm strips using a laboratory meat grinder. The moisture content of SCC was determined by uniform drying at 105 °C for ∼16 h to constant weight.

2.2. Microorganisms and enzymes

Trichoderma reesei RUT C-30 was grown on potato dextrose agar (DIFCO, Detroit, MI) at 30 °C for 4 days. *Lactobacillus plantarum* sp. 14431 and *Lactobacillus delbrueckii* NRRL-B-445 were grown on *Lactobacilli* MRS (deMan Rogosa Sharpe) agar (DIFCO, Detroit, MI) plates at 37 °C for 48 h. *Kluveromyces marxianus* IMB-3 was grown on YEPX agar plates (consisting of yeast extract, 10 g/l; peptone, 20 g/l; xylose, 20 g/l; agar, 20 g/l) at 30–32 °C for 48 h. Commercial cellulase preparation (Multifect B) was procured from Genencore (Rochester, NY).

2.3. Supplement media

2.3.1. Media for fungal cellulase production

Urea, 2 g/l; corn steep liquor (60% solid), 10 g/l; 10× mineral salts, 10 g/l. The mineral salts contained KH_2PO_4, 0.15 g/l; $(NH_4)_2SO_4$, 0.05 g/l; $MgSO_4$, 0.006 g/l; $CaCl_2$, 0.006 g/l; $FeSO_4{\cdot}7H_2O$, 0.00005 g/l; $MnSO_4{\cdot}H_2O$, 0.000016 g/l; $ZnSO_4{\cdot}7H_2O$, 0.000016 g/l; $CoCl_2{\cdot}6H_2O$, 0.000037 g/l [17]. The pH was adjusted to 6.0.

2.3.2. Media for lactic acid production by Lactobacilli

Yeast extract, 5 g/l; bacto peptone, 10 g/l; sodium acetate, 5 g/l; sodium citrate, 2 g/l; K_2HPO_4, 2 g/l; $MgSO_4{\cdot}7H_2O$, 0.58 g/l; $MnSO_4{\cdot}7H_2O$, 0.12 g/l; $FeSO_4$ $7H_2O$, 0.05 g/l; Tween 80, 1.0 g/l [9]. The pH was adjusted to 5.5–6.0.

2.3.3. Media for ethanol production by yeast

Filter-sterilized yeast nitrogen base, 1.7 g/l; urea, 2.27 g/l; and peptone, 6.56 g/l [13]. The pH was adjusted to 5.5–6.0.

2.4. Inoculum preparation

2.4.1. Inoculum for cellulase production

T. reesei RUT C-30 strain grown on PDA plate was inoculated into 50 ml medium in 300 ml Erlenmeyer flask containing potato dextrose broth, 24 g/l; Tween 80, 7.5 g/l; yeast extract, 1 g/l and incubated at 30 °C for 96 h, 200 rpm. The mycelia obtained were blended in 25 ml sterile water for 5 s in a Warring blender at 25 °C and was used as fungal inoculum.

2.4.2. Inoculum for lactic acid production

L. plantarum or *L. delbrueckii* was grown on MRS agar plates at 37 °C for 24–48 h. Cells on the surface of the MRS agar plates were washed on the surface with 5 ml of 10× supplement medium. The optical density of cell suspension was measured at 600 nm. The cell suspension was used as source of inoculum for lactic acid production aerobically.

2.4.3. Inoculum for ethanol production

K. marxianus IMB-3 was grown on YEPX agar plates for 72 h. The culture plates were washed with 5 ml of 10× supplement medium and used as source of yeast inoculum for ethanol production aerobically.

2.4.4. Liquid culture of SCC for cellulase production

Five grams of SCC (containing 60% moisture) was placed in a 300 ml Erlenmeyer flask, suspended in 40 ml of water and sterilized. After cooling, 5 ml of supplement nutrients and 5 ml of fresh *T. reesei* inoculum were added and incubated at 30 °C, 200 rpm for 10 days. Periodically, 1 ml samples were taken and centrifuged at 5000 rpm and clear supernatant was saved for overnight at 4 °C prior to enzyme assay.

2.4.5. Enzyme production from spent casings during SSC growth

The major carbohydrate component of sausage cellulose casing is ∼94% glucan, 1–4% xylan and negligible amounts of lignin [5] (Sreenath et al., unpublished). In addition to large concentration of cellulose, spent casings also contain meat juices, salt, nitrate, and nitrite [2]. The moist shredded casing waste of 4 cm × 0.4 cm strips (containing 60% moisture), was wetted with tap water to initial moisture content of 80% along with 0.1% Tween 80 and supplement media. Other agricultural substrates such as wheat bran, wheat straw, corncob and alfalfa were evaluated for SSC growth with or without blending with spent casings. The substrates were blended in 1:1 proportion on dry weight basis, Moistened substrate(s), 75 g, taken in a 1 l bioreactor was steam sterilized and inoculated by mixing 10 ml of starter culture inoculum of *T. reesei* RUT C-30 strain. The bioreactor was incubated at 32 °C for up to 8 days with 1 atm of moist air. For experiments on enzyme assays, the residue(s) are extracted with unsterilized tap water using a solid:liquid ratio of 1:3 for 90 min at 25 °C, followed by filtration. The clear supernatant solution is decanted and saved as enzyme at 4 °C prior to assay. Whole enzyme-rich substrate referred as "enzyme-rich casing" was stored at 4 °C for overnight prior to SSF.

2.4.6. Determination of enzyme activity

The filter paper cellulase activity of both commercial cellulase Multifect B and SSC in-house brewed cellulases were measured as described by Mandels et al. [18]. The protein content of the enzyme was determined by Lowry's reagent [19]. Two milliliters of reaction mixture consisting 1.0 mg enzyme protein, 50 mg strip of Whatman no. 1 filter paper and 0.05 M sodium phosphate buffer pH 4.8, was incubated at 50 °C for 1 h. The reducing sugar liberated was estimated by dinitrosalicylic acid reagent [20]. One IU of filter paper unit is defined as the amount of enzyme required to liberating 1 μmol of glucose under assay conditions. The level of filter paper cellulase (FPase) activity produced in SSC was expressed as IU/g dry weight of the substrate employed.

The CMCase activity was determined using 1.0 ml of 1% carboxymethyl cellulose 7LF in 0.05 M sodium acetate buffer pH 4.8 and 0.5 mg enzyme protein [21]. The reaction mixture was incubated at 50 °C for 30 min prior to reducing sugar estimation. One IU of CMCase is defined as the amount of enzyme that catalyzed the formation of 1 μmol glucose/min under assay conditions.

Similarly xylanase activity was assayed using 1.0 ml of 2% soluble oat spelt xylan in 0.05 M sodium acetate buffer pH 5.0 and 0.5 mg enzyme protein [15]. The reaction mixture was incubated at 50 °C for 30 min prior to reducing sugar estimation. One IU of xylanase activity referred to the amount of enzyme that catalyzed the formation of 1 μmol xylose/min under assay conditions.

2.5. SSF of fresh SCC

The SSF were conducted in 125-ml Erlenmeyer flasks in 50 ml final volume consisting 5 g of fresh SCC or other agricultural substrates on wet weight basis, suspended in distilled water, with 2.5 g $CaCO_3$ and sterilized for 20 min at 121 °C. The $CaCO_3$ is only used for buffering the lactic acid fermentation [9]. For ethanol fermentations, $CaCO_3$ was not added. The enzyme-rich casing with 50 FPase was directly mixed as such with 5 g of fresh SCC along with other components of fermentation medium. Cell inoculum of *L.* plantarum or *K. marxianus* IMB-3, 5 g/l was respectively added into SSF medium followed by addition of 5 ml of supplement media for *Lactobacillus* and yeast fermentation. The SSF was conducted at 41–45 °C, pH 5.5–6, at a shaker speed of 200 rpm for 2–5 days. At various intervals, 1 ml samples were taken and centrifuged at 16,000 × *g* for

3 min. The clear sample was saved at 4 °C for overnight for analysis of sugars, lactic acid and ethanol by high performance liquid chromatography. The amount of product formed in the fermentation media was expressed as g/l total mix. Note that the a liter of total mix includes 100 g of fresh SCC, enzyme-rich casing or blends, cells, supplement nutrients, and buffering salts of the medium.

2.6. Analyses

Dry weight of the enzyme-rich casing or blend was determined by drying them at 105 °C for over night to uniform weight for expression of SSC enzyme activity. Similarly, the amount of degradation of SCC was determined by drying the entire flask contents before and after SSF. The reducing sugars liberated during SSF were estimated by dinitrosalicylic acid method [20]. The sugars, organic acid and ethanol were determined by high performance liquid chromatography (HPLC) using ION-300 column (300 mm × 7.8 mm) (Interaction chromatography, San Jose, CA) with refractive index detector [9,13]. The mobile phase was 2.5 mM H_2SO_4 with a flow rate of 0.4 ml/min at 60–65 °C, and 4130–6200 kPa.

3. Results and discussion

3.1. SSC cellulase production from SCC

The levels of filter paper cellulase (FPase) and carboxymethyl cellulase (CMCase) production from SCC in submerged culture ranged from 5 to 7 IU/ml after 5–6 days of growth with *T. reesei* (data not shown). However, xylanase production was higher and in the range of 19–20 IU/ml. Cumba and Bellmer [5] similarly reported lower cellulase production in submerged fermentation from SCC producing 1.3 IU/ml FPase after 7 days. Cellulase production by submerged fermentation and SSC has been reviewed using various microorganisms, substrates and nutrients [22–29]. Preliminary studies have shown that SSC can be used to manufacture enzymes at significantly lower costs than those encountered with conventional submerged culture [26–28].

During SSC, the rapid growth of *T. reesei* proved the utility of SCC for cellulase production either alone or in combination with other substrates such as wheat bran, wheat straw, alfalfa and corncob. Synergistic cellulase production was noticed when SCC blended with alfalfa or wheat bran compared to cellulase production alone on these substrates. These substrates alone gave 20–64 FPase/g and 34–74 CMCase/g biomass. The blends of SCC with wheat bran or alfalfa after SSC produced 152 FPase/g and 170 CMCase/g biomass (Fig. 1A and B). Fig. 2 also shows enzyme production patterns in these substrates during 4- and 8-day harvest. The 8-day harvest generally produced higher cellulase than 4-day harvest.

Other agriculture substrates such as wheat straw alone produced 38.5 CMCase and 53.1 FPase during SSC whereas corncob produced 37.4 CMCase and 29.6 FPase on 8-day harvest. However, combination of SCC + wheat straw or corncob failed to show synergistic cellulase production.

The amount of SSC xylanase produced was 1.5–2 times higher with SCC alone and blends of SCC + wheat bran on 4-day of harvest (Fig. 1C). Increase in xylanase production was also found in blends of SCC + alfalfa on 8-day of harvest. These results prove previous reports of pure cellulose stimulating xylanase production by fungi [30–32].

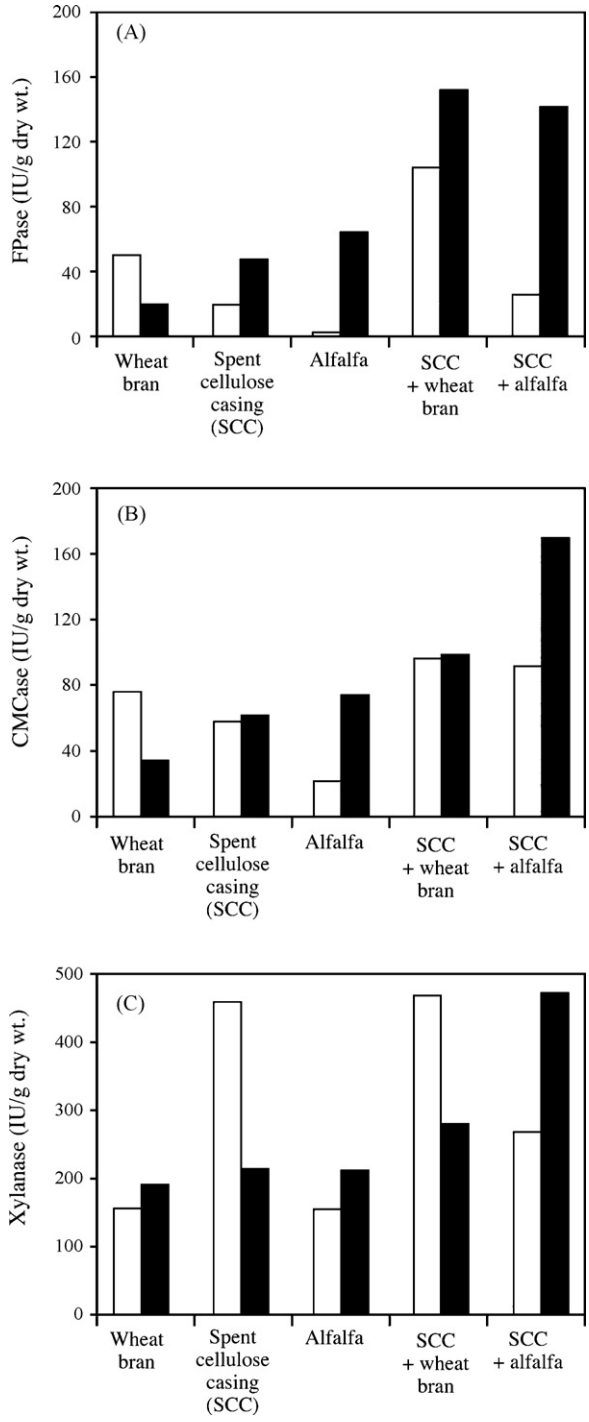

Fig. 1. SSC of *T. reesei* on SCC and other agricultural substrates for enzyme production: (A) filter paper cellulase; (B) carboxymethylcellulase; (C) xylanase ((□) 4-day cultivation; (■) 8-day cultivation).

3.2. SSF lactic acid and ethanol production from fresh SCC using enzyme-rich substrate residue

Table 1 summarizes fermentation conditions employed in lactic and ethanol production. The enzyme-rich casing or casing blends with 50 FPase was directly mixed with 5 g of fresh SCC in 50 ml of fermentation medium. This approach of SCC bioconversion employing enzyme-rich substrate in SSF without

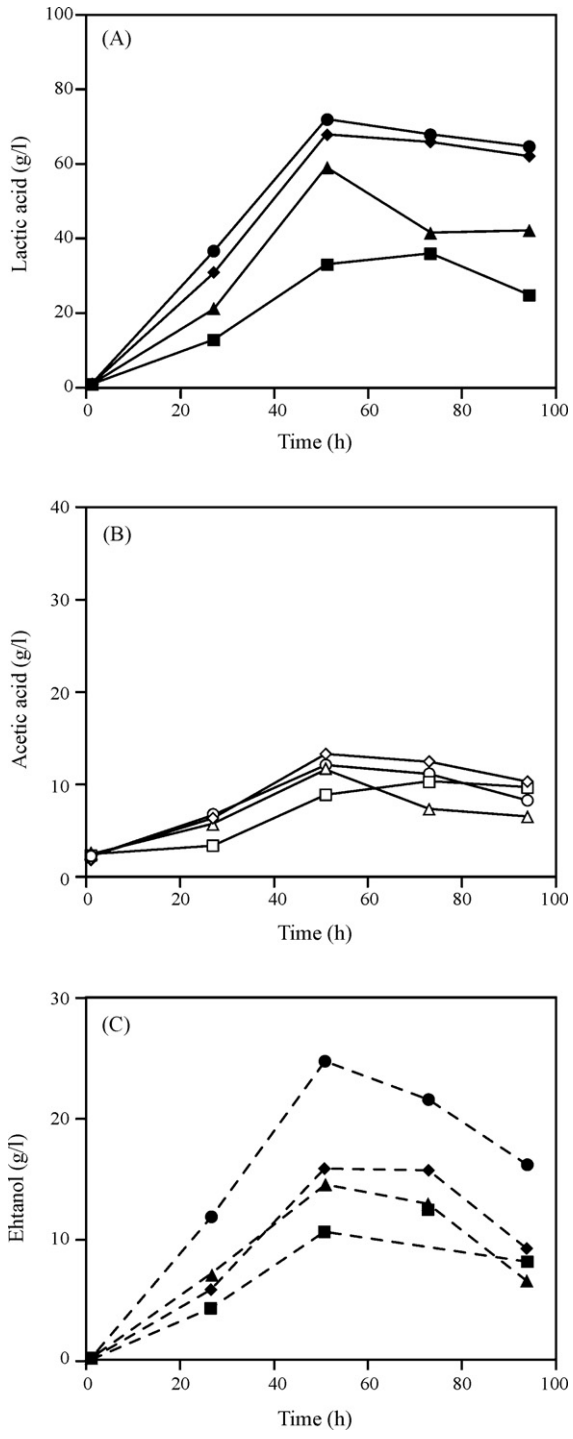

Fig. 2. Fermentation of fresh SCC using various SSC enzyme-rich substrates. (A) Lactic acid production by *L. plantarum*: SCC (●); SCC + wheat bran (♦); SCC + wheat straw (■); wheat bran + corncob (▲). (B) Acetic acid production by *L. plantarum*: SCC (◯); SCC + wheat bran (◊); SCC + wheat straw (□); wheat bran + corncob (△). (C) Ethanol production by *K. marxianus*: SCC (●); SCC + wheat bran (♦); SCC + wheat straw (■); wheat bran + corncob (▲).

enzyme extraction and separation saved considerable processing time and laborious methods. The fermentation temperature of 41–45 °C was required for simultaneous saccharification of fresh substrate and growth of fermentation organism for maximum conversion [8,9,16,33–35]. This temperature is advantageous in terminating the growth of cellulase producing fungus. The lactic fermentation medium was buffered using $CaCO_3$; buffering was not required for ethanol fermentation medium (Table 1). In this SSF, more than 90% of SCC was saccharified and the resulting sugars were converted to high rates of lactic acid or ethanol with in a period of 50 h (Fig. 2). Both Cumba and Bellmer [5] and Sanders et al. [7] have reported more than 80% degradation of spent cellulose casing after 24–48 h of cellulolytic saccharification employing various commercial enzyme preparations.

Table 1
Fermentation conditions for SSF lactic acid and ethanol production

Fermentation parameters	*L. plantarum*	*K. marxianus*
Working volume (ml)	50	50
Temperature (°C)	41	45
pH	5.5–6.0	5.5
Agitation (rpm)	180	180
O_2 (mmol/l)	9.2	9.2
Initial cells (g/l)	5 (or 20 OD)	5 (or 20 OD)
SCC (g)	5	5
Cellulase (FPU)	50	50
Nutrients (g/l)	MRS broth[a]	Supplements[a]
Ca CO_3 (g/l)	5	Absent

[a] Refer Section 2.3.

The 8-day-old enzyme-rich casing was employed in SSF of fresh SCC (Fig. 3). The enzyme-rich casing contained 48 FPase/g and 62 CMCase/g substrate and that of blends of SCC + wheat bran which showed 152 FPase/g and 170 CMCase/g substrate. The SSF of fresh SCC with *L. plantarum* or *K. marxianus* showed complete solubilization of SCC at ∼50 h with these enzymes. *L. plantarum* produced ∼70 g/l lactic acid in 50 h with both enzyme-rich SCC and blend of SCC + wheat bran (Fig. 2A). The lignocellulosic components in agricultural residues need to be pretreated to release cellulose, the primary inducer of the cellulase production [5,36]. However, the purity of cellulose in SCC possibly helps in inducing pure cellulolytic material. These pure cellulases degrade fresh SCC rapidly due to enhanced saccharification and fermentation. Hence, enzyme-rich casing with lower cellulase activity producing a higher lactic acid rates similar to the blends of SCC + wheat bran with higher cellulase activity.

The other blends of enzyme-rich casing such as SCC + wheat straw and SCC + corncob produced 35.6 and 59 g/l lactic acid and 14.1–17.4 g/l acetic acid, respectively, after SSF of fresh SCC employing *L. plantarum* (Fig. 2A and B). *K. marxianus* IMB-3 produced 25 g/l ethanol in 50 h of SSF with enzyme-rich casing (Fig. 2C). Whereas the other blends of enzyme residues produced 10.6–15.8 g/l ethanol.

3.3. Effect of enzyme harvest on SSF of SCC

The enzyme harvested on 8-day produced 10–20% higher lactic acid compared to 4-day harvested enzyme (Fig. 3A). The ethanol production was slightly higher in the 8-day enzyme harvest compared to 4-day enzyme (Fig. 3B). The enzyme

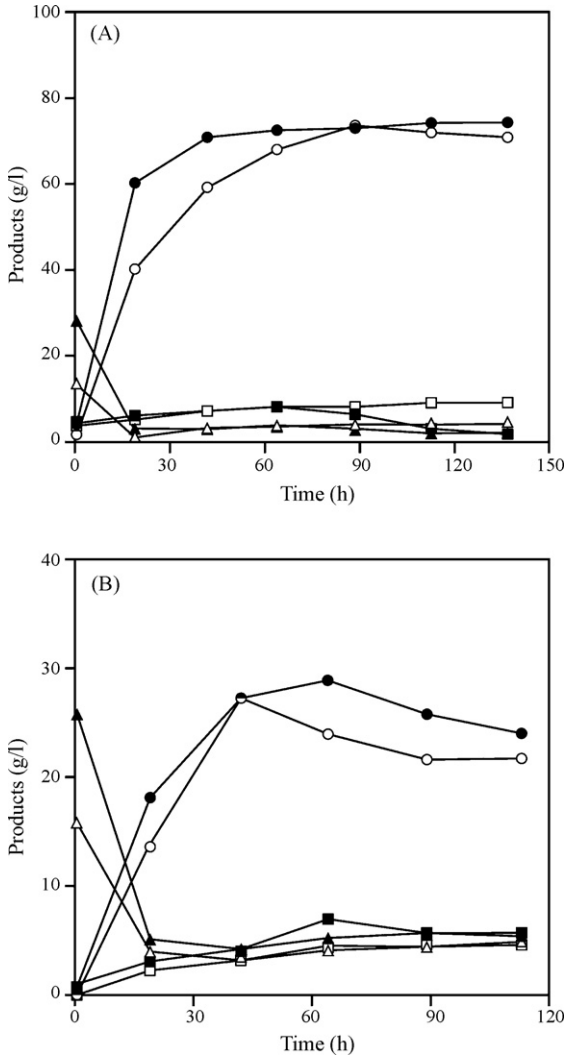

Fig. 3. Effect of SSC harvest time of SCC grown enzymes on fermentation of fresh SCC. (A) Lactic acid production by *L. plantarum*—8-day harvest: lactic acid (●), acetic acid (■), reducing sugar (▲); 4-day harvest—lactic acid (◯), acetic acid (□), reducing sugar (△). (B) Ethanol production by *K. marxianus*—8-day harvest: ethanol (●), acetic acid (■), reducing sugar (▲); 4-day harvest—ethanol (◯), acetic acid (□), reducing sugar (△).

harvested at 8-day contained higher amounts of level of filter paper cellulase (FPase) than 4-day enzyme. However, the amounts of acetic acid produced were lower in both 4- and 8-day harvested enzyme (Fig. 3A and B). The fermentative efficiency of *Lactobacillus* and *Kluveromyces* for lactic acid and ethanol production, respectively, enhanced due to increased compatibility between cellulolytic saccharification of the fresh substrate and the growth temperature of the microorganisms [8,9,16,33–35]. The reducing sugars of cellulolytic saccharification of SCC contained mainly glucose, cellobiose and xylose [5] (Sreenath et al., unpublished). The saccharified reducing sugars did not accumulate in the fermentation medium as they were simultaneously utilized rapidly for lactic acid and ethanol production. At this point it is not known that there are any inhibitory or stimulatory effects of meat juices in the SSF fermentation medium in producing these metabolites.

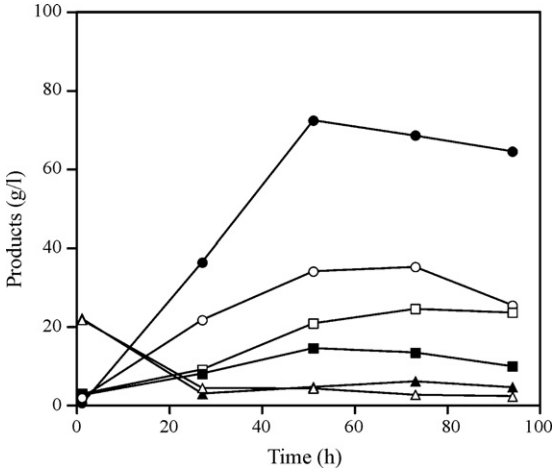

Fig. 4. Comparison of lactic acid production from fresh SCC in *L. plantarum* and *L. delbrueckii* using SCC grown enzymes. *L. plantarum*: lactic acid (●); acetic acid (■); reducing sugar (▲). *L. delbrueckii*: lactic acid (◯); acetic acid (□); reducing sugar (△).

3.4. SSF lactic acid production in *L. plantarum* and *L. delbrueckii*

The lactic acid production by *L. plantarum* was twice more than that of *L. delbrueckii* using enzyme-rich casing (Fig. 4). *L. plantarum* produced 70 g/l lactic acid and 14.7 g/l acetic acid in 51 h, whereas *L. delbrueckii* produced 35.4 g/l lactic acid and 24.7 g/l acetic acid in 73 h from fresh SCC (Fig. 4).

3.5. SSC enzymes versus commercial enzyme in SSF conversion of SCC

The commercial cellulase preparation and SSC enzyme-rich casing with 25 FPase when directly mixed with 5 g of fresh SCC in 50 ml of SSF medium produced similar amounts of lactic acid (Fig. 5). However, the amount of SSF ethanol produced

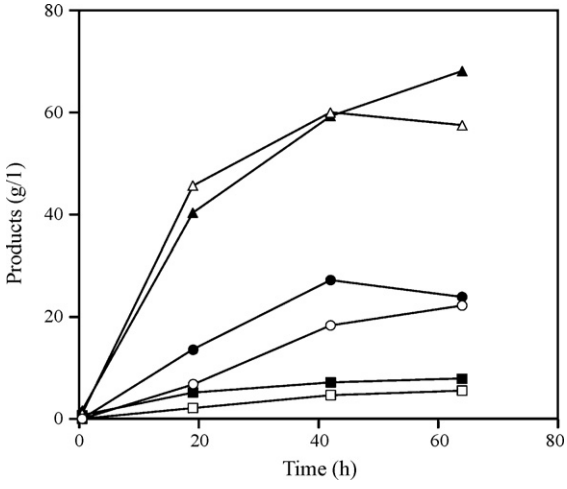

Fig. 5. Comparison of SCC grown enzymes with commercial cellulase preparation on fermentation of fresh SCC using *L. plantarum* and *K. marxianus*. Enzyme-rich SCC: lactic acid (▲); acetic acid (■); ethanol (●). Commercial cellulase preparation: lactic acid (△); acetic acid (□); ethanol (◯).

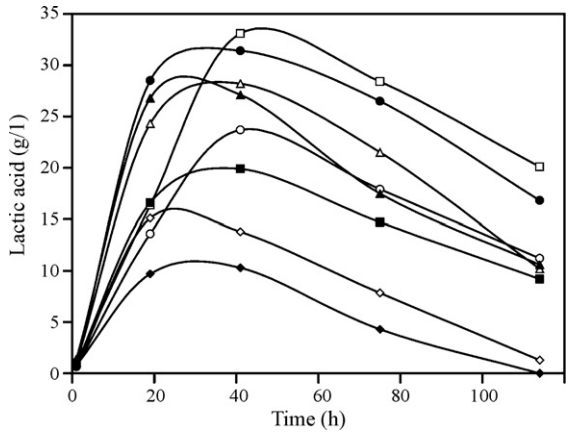

Fig. 6. Lactic acid production from various agricultural substrates using enzyme-rich SCC. Alfalfa (◯); beet pulp (□); soya fiber (△); corncob (◊); corn stover (●); wheat straw (■); wheat bran (▲); cranberry pomace (♦).

was slightly higher in SSC enzyme-rich casing than commercial enzyme preparation.

3.6. Lactic acid production from un-pretreated feedstock residues

Additionally the efficacy of enzyme-rich casing residue was tested for lactic acid production from various non-pretreated agricultural feedstocks residues such as corn stover, wheat bran, wheat straw, Soya fiber, alfalfa and beet pulp. The SSC enzyme-rich casing with 25 FPase when directly mixed with 5 g of various agricultural substrates in 50 ml of SSF medium produced lactic acid in the range of 25–35 g/l by *L. plantarum* in 41 h (Fig. 6). Similar reports are available on lactic acid production from other agricultural feedstock residues and wastes employing commercial enzymes [8,9,16,37]. The reducing sugar release in most of the substrates was sporadic in the first 1 h of SSF and the range was 18–30 g/l. At 41 h, the lactic acid productivity reached peak in most of the substrates with reducing sugar release decreasing to 6–17 g/l suggesting higher sugar consumption. The lactic acid production was considerably reduced after 3–4 days of SSF and 5–8 g/l of reducing sugars was still left in the medium unfermented. The fermentation stopped probably due to unfavorable fermentation conditions due to depletion of nutrients and low pH causing cell death [8,14]. Fermentation of herbage biomass requires efficient saccharification of fiber, which normally involves various pretreatment procedures. These pretreatments include treatment with acids or bases coupled with elevated temperatures or softening the fiber with steam or liquid ammonia [8,13,35,36]. In this study, without pretreatment, the beet pulp and corn stover gave higher lactic acid production and wheat bran and alfalfa ranked next. However, additional research is necessary on large-scale optimization of bioconversion of spent cellulose casings and its commercial feasibility.

4. Conclusion

Spent cellulose casings, a waste product of sausage industry consisting of pure cellulose polymer, alone or when blended with other substrates produced high yields of cellulases on solid support. These cellulases without laborious extraction directly hydrolyzed and saccharified fresh spent cellulose casing wastes and other agricultural residues extensively and simultaneously fermented to produce high yields of lactic acid and ethanol. This bioconversion process has potential to reduce the cost of disposal (over conventional landfill) to sausage producers, as well as create value-added products and co-products.

Acknowledgements

This work was funded by Industrial and Economic Development Research (I & EDR), UW-Madison, WI and USDA ARS-Dairy Forage Research Center, Madison, WI. The authors are thankful to Mrs. Holalkere V. Geetha for her excellent technical support and Dr. Thomas W. Jeffries for providing facilities at Forest Products Laboratory, Madison, WI. This work was presented in 227th ACS symposium, Anaheim, California, 28 March–1 April 2004.

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